Modeling bacterial survival in unfavorable environments

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SUMMARY

The long-term survival of pathogenic microorganisms was evaluated and modeled in simulated fermented and dried, uncooked sausages, such as salami and pepperoni. *Listeria monocytogenes* and *Salmonella* were inoculated in BHI broths with added lactic acid or lactate (0–1.5%), NaCl (0–19%) and $NaNO_2$ (0–200 ppm) and then incubated at 4–42 °C for up to 9 months. Enumerations of surviving cells showed several forms of declining curves, including classic first-order declines, shoulder or lag phases, and two-phase declines with shoulder. Two primary models were tested for their ability to depict the data. The effect of the environmental conditions on the parameters of the models were described with multiple regression equations (secondary models).

INTRODUCTION

Small populations of pathogenic bacteria sporadically appear in non-sterilized foods due to the raw ingredients or by contamination during processing operations. The ability to predict whether these pathogens will grow, survive or die is important to food processors and regulatory agencies. Development of growth models has been an active area of food microbiology and these models are evident in the presentations and posters given at this symposium. The first microbiological models to be developed, however, were for lethal treatments, primarily heat. The first-order reduction in viable cells with time has been the basis for thermal processing since the 1920s. Despite universal reliance on first-order or death kinetics for D value calculations, more complex survival patterns are frequently observed [12,15, 16,21,23]. Expansions of the thermal processing model were presented earlier in this session [14,18] and in the literature [10,15,16].

The slow decline in microbial numbers during storage has not received quantitative treatment. It would be desirable to design a food in which small numbers of a particular pathogen would not only fail to increase in numbers but actually decrease to undetectable levels. This would be particularly valuable for foods which are not thermally processed or have a shelf-life dependent primarily upon refrigeration. Shelf-stable and semi-stable meat products such as pepperoni, some salami, Westphalian hams and Proscuito are examples of foods where survival of pathogens is of concern and are the foci for the work presented in this paper. Other foods where pathogen survival is important are cheeses, refrigerated ready-to-eat and microwaveable products.

The inactivation of microorganisms in water, soil and ground water has been described by equations with a constant value followed by a first-order decline [3,5,8]. Conditions permitting long-term survival of plant pathogenic bacteria include: 1) to have an association with living or dead plant tissues; 2) to be in bacterial aggregates or otherwise protected; and 3) to be in a state of reduced metabolism [8]. Similar conditions are found in food products.

Because the inhibition of microorganisms followed by slow reduction in microbial numbers depends on the interactions of several factors [13], the intent of this ongoing study was to develop models that describe the decreases in bacterial populations during storage at various temperatures in media containing varying additions of lactic acid, sodium chloride and sodium nitrite. Multiple regression equations that describe the expected values of the parameters for specific environmental conditions were then calculated. Predicted rates of inactivation from these equations were then compared to literature data of bacterial survival in foods. *Listeria monocytogenes* and *Salmonella* were chosen for modeling because of their known occurrence in meat products. The principle objective of this presentation is to introduce primary models [24] for describing the inactivation/survival of microorganisms. Preliminary calculations for the secondary regression models will be used to show the general relationship of the environmental parameters to survival and to compare the models with published inactivation rates in foods.

Models

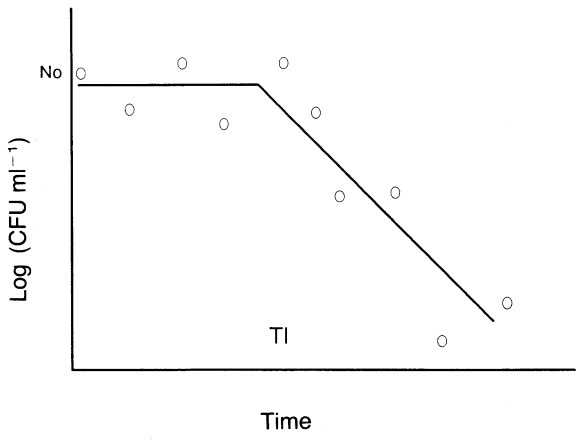
Experimental trials indicated that survivor curves exhibited lag periods or shoulders, ie., time periods where the

bacterial populations remained at the inoculation level. After the shoulder period, the logarithm of the bacterial numbers generally declined linearly with time (first-order). In some instances a subpopulation of more persistent bacteria was observed that declined at a slower rate than the majority of the cells (tailing).

Two primary models were developed to describe the declining numbers of microorganisms with time. The linear model had the population equal to the initial population until the end of the shoulder period (Fig. 1). After that time, the population (log CFU ml^{-1}) decreased linearly with the slope equal to $-1/D$, where D is the classic time for a one log decrease. This model did not attempt to account for a subpopulation.

The second model was derived from the logistic-based model of Kamau et al. [7] to include a shoulder and two populations (a major population and a subpopulation) [11] (Fig. 2). The respective D values for the linear portions of the curve were equal to 2.3/b, tl was the shoulder period and F1 the proportion of cells in the major population. If no subpopulation existed, F1 equaled 1 and the right side of the equation was zero. This resulted in a shoulder and single decreasing slope. If, in addition, no shoulder was present, tl would be zero and the equation would essentially yield a straight line.

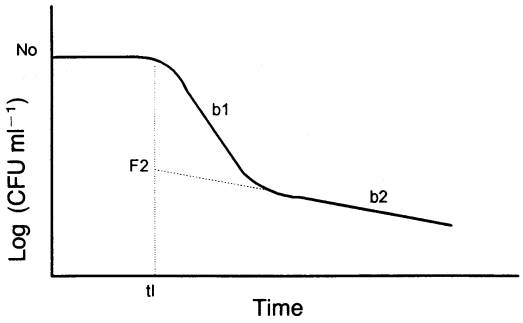
Both of these models were installed in a Gauss-Newton curve fitting program (ABACUS Software Program, ERRC, USDA, Philadelphia, PA) to calculate the values of the respective parameters for each survivor curve. Changes in the value of each parameter with changes in the environmental factors were then described by second-order multiple regression equations (secondary models) using the RS/1 statistical program (BBN Software Products Corp., Cambridge, MA).



$$Y = No \quad 0 < t < Tl$$

$$Y = No - (1/D)(t - Tl) \quad t > Tl$$

Fig. 1. Equations and diagram of linear model.



$$LOG \frac{N}{No} = LOG \left[\frac{F1(1 + e^{-b1*tl})}{(1 + e^{b1(t - tl)})} + \frac{(1 - F1)(1 + e^{-b2*tl})}{(1 + e^{b2(t - tl)})} \right]$$

b1 = maximum specific death rate of major population

b2 = maximum specific death rate of subpopulation

F1 fraction of initial population in major population

(1 - F1) F2 fraction of population in subpopulation

tl shoulder or lag period

t time

N population (log CFU ml^{-1}) surviving at time = t

No initial population (log CFU ml^{-1}) at time = 0

D value is given by D = 2.3/b for each population

Fig. 2. Equation and diagram of logistic model.

Microbial techniques

Brain heart infusion (BHI) broths were varied by additions of 0–19% NaCl and 0–200 ppm $NaNO_2$. Lactic acid (0–1.5%) was added to give pH values from 7.2 to 3.5, respectively. Overnight cultures of three strains of *L. monocytogenes* (HO_YJ_S, V-7, Scott A) were grown and combined. Flasks of BHI broths were inoculated with 10^7–10^8 CFU ml^{-1} and stored at temperatures from 4 to 42 °C. 249 flasks were sampled at appropriate intervals and the survivors determined by plating on TSA (Spiral Systems Inc., Cincinnati, OH). Three strains of *Salmonella* (*S. dublin, S. typhimurium*, food isolate) were similarly grown, inoculated into 186 flasks of BHI broths, stored, and survivors determined.

Survival curves

The survival of both species varied from less than 1 h to over 6 months. For *Salmonella*, D values of the major population ranged from 0.02 to 146 days depending on the treatment. Shoulder periods were evident in 18% of the treatments and ranged from 0 to 56 days. However, a consistent pattern for the shoulder periods was not evident making it impossible to develop a useful regression equation. The occurrence of the subpopulation was likewise inconsistent. Subpopulations were present in 23% of the treatments and usually comprised about $1/10^4$ of the original cells. For

Listeria, D values ranged from 0.38 to 837 days, shoulder periods were evident in 13% and subpopulations were observed in 15% of the flasks.

Two approaches were taken to develop secondary models for these data. In the first, all of the curves having shoulder periods were refitted to a simple linear decline with no shoulder or subpopulation. This was done with both the linear and logistic models. Multiple regression equations were calculated for the simple D values. The second approach was to determine the time for a 4 log reduction in population. This reduction would eliminate the numbers of a pathogen likely to be present in products made under good manufacturing practices. This also removed the subpopulation, if present, from consideration at this time. The logistic model with shoulder was solved for time (Fig. 3) and the time for a 4 log reduction was calculated from the parameter values for each flask.

The initial regression equation calculations for *Salmonella* survival using the logistic model gave the best fits for log of the simple D values and for the log of time for a 4 log reduction (Fig. 4). Both r^2 values were 0.83 and the times for 4 log reductions calculated by the two equations were very similar. An initial examination of the *Listeria* survival data showed a range of 1.6 to over 1000 days and an r^2 of 0.86 for the regression of the explanatory variables vs the logarithm of the time for a 4 log decline.

Figures generated from the regression equations (logistic model, time for 4 log decline) illustrate the influence the four variables had upon survival (Figs 5 and 6). Survival decreased rapidly as the temperature increased. *Salmonella* had very long survival at 5 °C. The longest survival was at pH 6 for *Listeria* and pH 6.5 for *Salmonella*. *Listeria* had its best survival between 6 and 10% NaCl and declined more rapidly below and above this range. *Salmonella* had maximum survival below 5% NaCl (Fig. 7). Nitrite in the BHI broths reduced survival of both microorganisms by 50% when increased from 0 to 30–40 ppm $NaNO_2$. For most conditions, *Listeria* exhibited longer survival times than *Salmonella*. The effect of temperature on the relative rate of inactivation (1/D) exhibited a reasonable fit to the Arrhenius relationships for the actual *Salmonella* data at pH 7.2, 6% NaCl and 15 ppm nitrite (Fig. 8).

A critical assumption was that the survival data starting with 10^7 CFU ml^{-1} gave the same inactivation rates as with lower inoculum levels representing more realistic populations

$$t = \frac{\ln\left[\frac{(1 + e^{-b1*tl})}{0.0001} - 1\right] + b1*tl}{b1}$$

Fig. 3. Equation of time for 4 log decrease using logistic model with shoulder and major population.

SALMONELLA INACTIVATION

n = 249

LOG (T4D) (DAYS) = -10.76 + 0.0102TEMP + 0.0702NaCl -0.0064NO2
+ 4.144pH -0.000039TEMP*NaCl - 0.000083TEMP*NO2
- 0.0097TEMP*pH + 0.000048NaCl*NO2 - 0.0108NaCl*pH
- 0.000038NO2*pH + 0.00014$TEMP^2$ - 0.0025$NaCl^2$
+0.000016$NO2^2$ - 0.303pH^2

R^2 = 0.83

LOG (D1) (DAYS) = -11.35 + 0.012TEMP + 0.068NaCl - 0.0066NO2
+ 4.123pH - 0.000039TEMP*NaCl - 0.000082TEMP*NO2
- 0.0099TEMP*pH + 0.000050NaCl*NO2 - 0.0106NaCl*pH
- 0.000027NO2*pH + 0.000123$TEMP^2$ - 0.00246$NaCl^2$
+ 0.000017$NO2^2$ - 0.301pH^2

R^2 = 0.83

LISTERIA INACTIVATION

n = 186

LOG (T4D) (HR) = -5.824 + 0.00470TEMP + 0.0521NaCL - 0.0255NO2
+ 3.185pH - 0.00170TEMP*NaCl + 0.000139TEMP*NO2
+ 0.00446TEMP*pH - 0.000072NaCl*NO2 + 0.00714NaCl*pH
+ 0.00152NO2*pH - 0.00115$TEMP^2$ - 0.00375$NaCl^2$
+ 0.000053$NO2^2$ - 0.274pH^2

R^2 = 0.86

Fig. 4. Regression equations for the inactivation of *Salmonella* and *L. monocytogenes*.

in foods. Therefore three treatments were inoculated with 10^3–10^8 *Listeria* per ml and the slopes of the decreasing cells were found to be parallel. These data were fitted with both the linear and logistic models; the D values for the former were slightly longer than the latter, but differences were less than the variation between inoculum levels. Overall, the D values were unaffected by inoculum sizes from 4.2 to 8.5 log (CFU ml^{-1}).

The interrelationship between pH and lactate anion concentration was explored because the buffering capacity of the BHI broth was less than that of a fermented sausage. Both *Listeria* and *Salmonella* growth were inhibited by lactate in the media. Data from 12 lactate buffers (3 pH values × 4 molarities) in BHI broths showed similar inactivation rates for *Listeria* at pH 7 with 0.1–1.0 M lactate. At pH 5, all inactivation rates were more rapid than at pH 7 and 0.5 M lactate was much faster than 0.1 M. An interesting relationship was observed: the log of the time for 4 logs inactivation was inversely correlated with the square root of the concentration of undissociated lactic acid (r^2 = 0.96).

Some of the most favorable pH-salt-nitrite-temperature treatments supported growth instead of death. These treatments were omitted from the calculations of the regression equations. However, it would be necessary to determine whether a specific combination of factors would permit growth before unknowingly inserting that condition into the

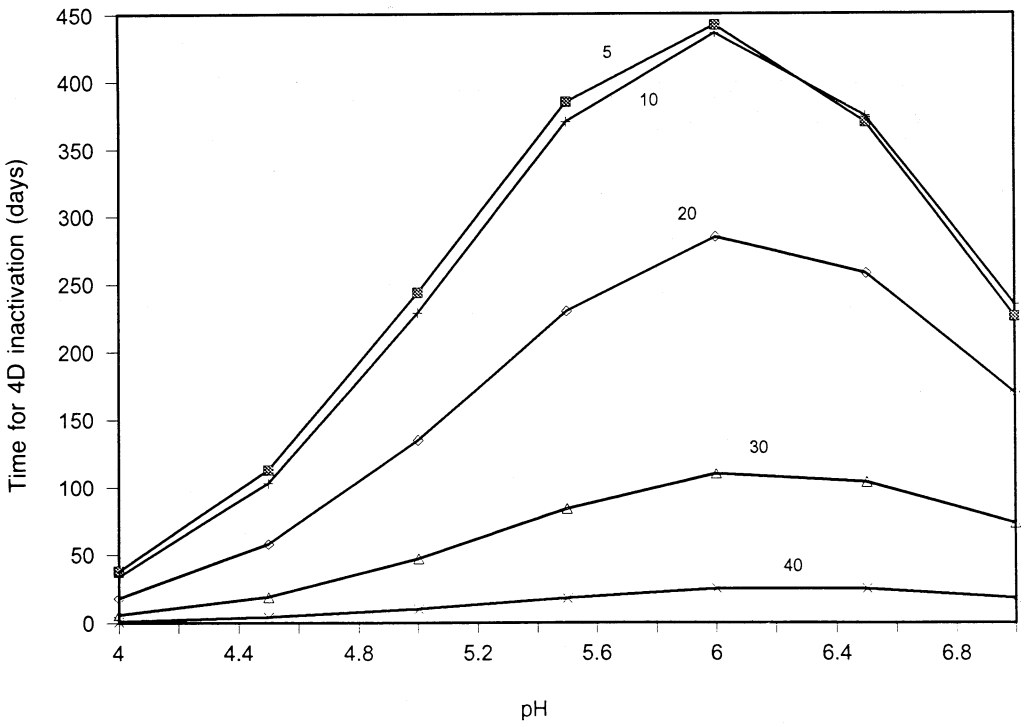


Fig. 5. Effect of temperature and pH on the calculated times for 4 log decrease of *Salmonella*. The other parameters were set at 9% NaCl and 0 ppm $NaNO_2$.

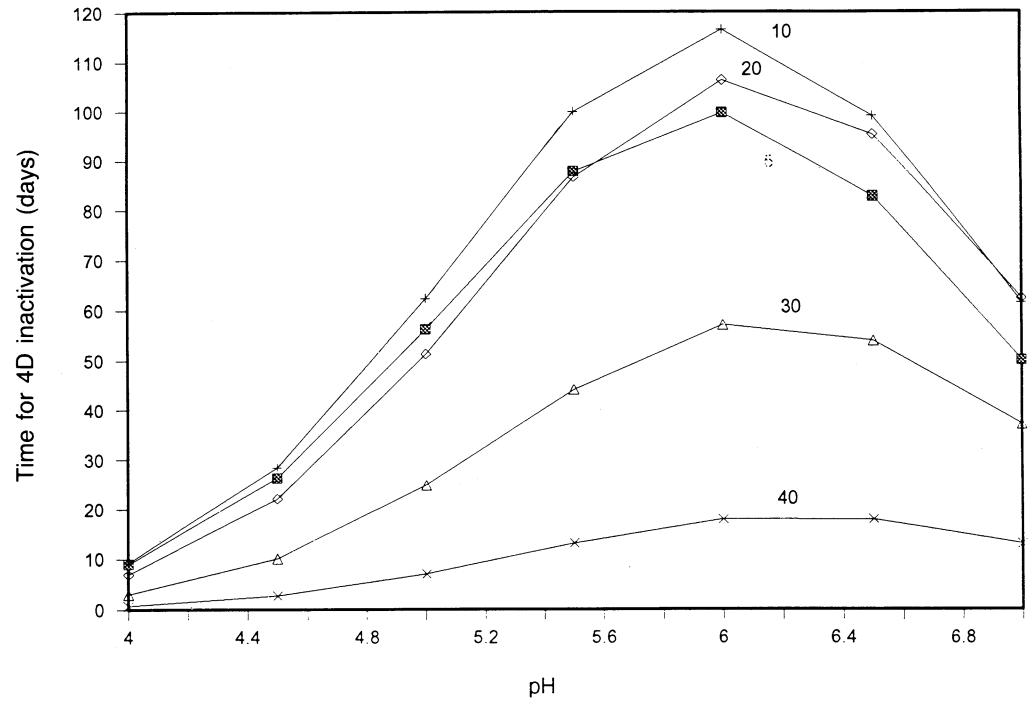


Fig. 6. Effect of temperature and pH on the calculated times for 4 log decrease of *Listeria*. The other parameters were set at 9% NaCl and 0 ppm $NaNO_2$.

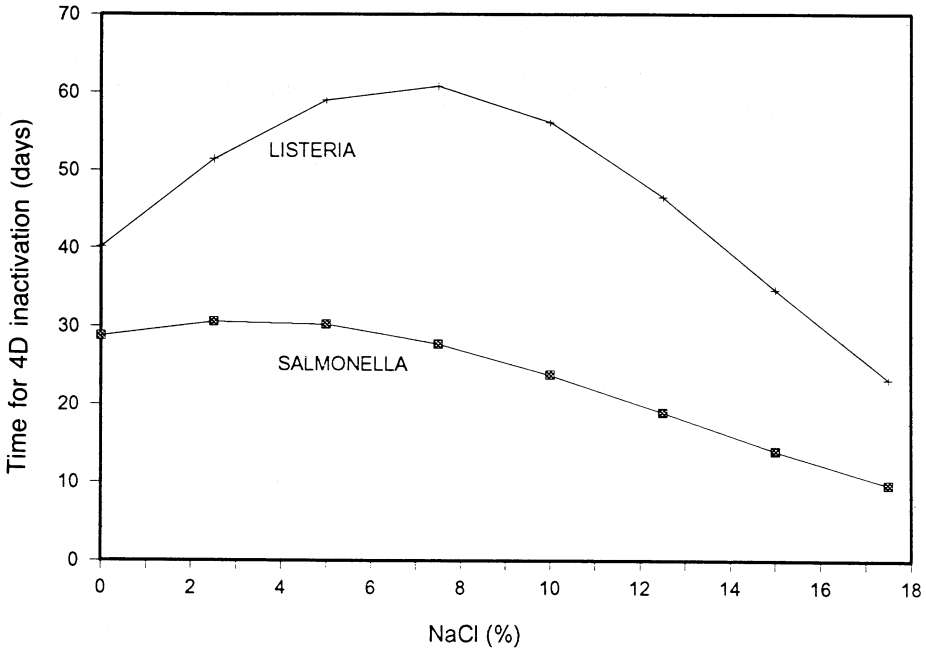


Fig. 7. Effect of added NaCl on calculated declines in *Salmonella* and *Listeria*. The other parameters were set at 20 °C, pH 6.0 and 25 ppm $NaNO_2$.

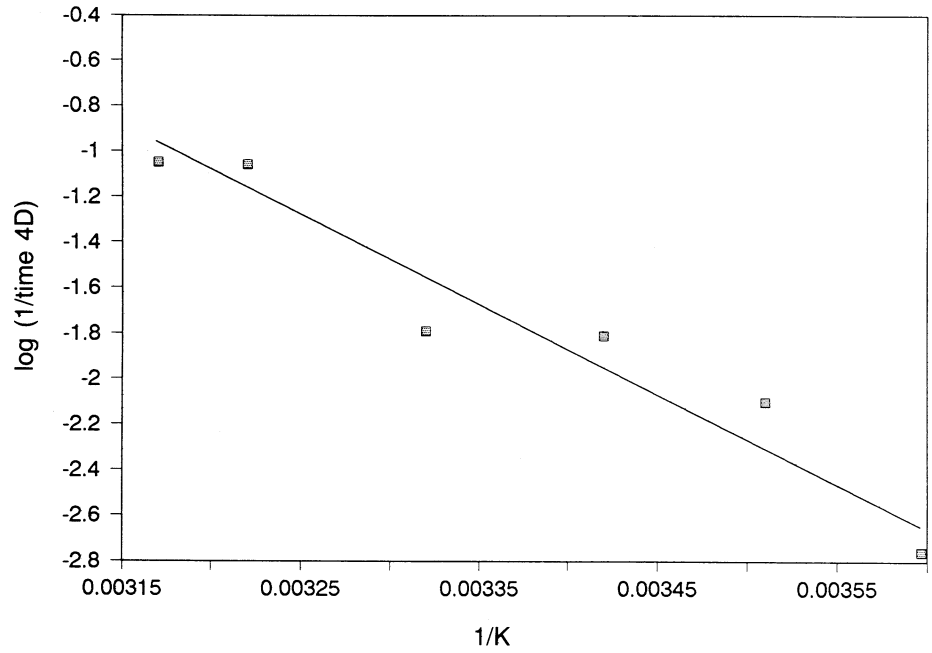


Fig. 8. Arrhenius relationship for the decline in *Salmonella* at pH 7.2, 6% NaCl and 15 ppm $NaNO_2$.

survival equations. Unfortunately, comparing the predicted growth of *Salmonella* using the Gompertz equation [4] with the *Salmonella* inactivation equation presented in this paper did not necessarily indicate whether growth or decline would occur. In some cases, a brief period of growth occurred followed by decline from the larger population. It may be possible that inoculum size was a factor when conditions favored growth. Growth and inactivation were greatly affected by temperature (assuming other factors were not extreme). High temperatures promoted both faster growth and faster inactivation.

Comparison of expected survival from these models with survival in inoculated food studies demonstrated that these models are reasonable first estimates of survival and worthy of further development (Table 1). Actual declines of *Listeria* were faster in the coleslaw than expected. The fermented sausage had no change in counts for 20 days while the model and the salami data agree on a relatively short survival.

TABLE 1

Validation of *Listeria* and *Salmonella* models

	TEMP	pH	NaCl	NO_2	D1 Measured (Days)	D1 Model (Days)
Listeria monocytogenes						
Coleslaw (vinager)	4	5	(0)	(0)	5	12
[2]	15	6	(0)	(0)	3	12
Salami	11	5.1	3.5	70	5	4
[22]						
Fermented sausages	20	4.6	2.4	85	N.C. 20	1
Frankfurters	4	(5.9)	2.2	26	GREW	14
[1]						
Cottage cheese	4	4.6	1	(0)	10	10
[6]						
Trappist cheese	10	5.4	1.1	(0)	N.C. 90	20
[9]					[30]	
Cheddar cheese	6	5.1	1.6	(0)	20	20
[17]						
Salmonella						
Pepperoni	12	4.6	7	(25)	9	5
[19]		5.0			15	13
		5.8			N.C.	44
Lebanon bologna						
[20]						
Fermentation	35	4.5	3	(25)	1	1

N.C. = No change in population for time period specified.
( ) Values not given in reference.

Frankfurters supported growth while the model predicted a slow decline. Perhaps frankfurters were a situation where growth would be followed by decline. The Trappist cheese had growth and decline over 90 days with no net change. However, the rate during the declining period was close to that predicted. Excellent agreement between measured and expected times were found for cottage cheese and cheddar cheese.

Two studies of *Salmonella* inactivation in fermented meats showed good agreement during storage of pepperoni made to have three pH levels [19] and during a 4-day fermentation period for Lebanon bologna [20].

CONCLUSION

This paper described two primary models that are capable of fitting survival/inactivation data that exhibit a lag or shoulder period followed by declining numbers of viable microorganisms. The second model includes resistant subpopulations. Changes in the parameter values of the models with changing environmental parameters were then described by regression equations (secondary models). Comparisons of D_1 values calculated by this model to literature D values demonstrated that inactivation modeling is feasible. However, refinements and reconciliation with growth models are needed.

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